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14. ABSTRACT
Two isoforms of sphingosine kinase (SphK) catalyze the formation of sphingosine-1-phosphate (S1P). Whereas, SphK1 stimulates cellgrowth and survival, it was found that when overexpressed in mouse NIH 3T3 fibroblasts SphK2 enhances caspase-dependent apoptosis inresponse to serum deprivation, independently of S1P receptors. Sequence analysis revealed that SphK2 contains a 9 amino acid motifsimilar to that present in BH3-only proteins. Studies showed that the BH3-only domain and catalytic activity contribute to the apoptoticeffects of overexpressed SphK2. Further studies in human carcinoma cells showed that overexpression of SphK2 increased the expressionof the cyclin dependent kinase (cdk) inhibitor p21, but interestingly had no effect on p53 or its phosphorylation. Correspondingly,downregulation of endogenous SphK2 with small interfering RNA (siRNA) targeted to unique mRNA sequences decreased basal anddoxorubicin-induced expression of p21 without affecting p53. In addition, downregulation of SphK2 decreased G2/M arrest in response todoxorubicin. Surprisingly however, siSphK2 markedly enhanced apoptosis induced by doxorubicin in MCF7 cells. This result raises thequestion of how overexpression of SphK2 decreases cell growth and enhances apoptosis while its downregulation sensitizes cells toapoptosis. A partial answer may come from the possibility that when SphK2 is overexpressed it does not always have the same subcellulardistribution as the endogenous protein. It may also be possible that proteolysis of overexpressed SphK2 might induce apoptosis due toliberation of its BH3 peptide domain, which does not occur at the levels at which endogenous SphK2 is expressed. Collectively, theseresults demonstrate that endogenous SphK2 is important for p53-independent induction of p21 expression by doxorubicin and suggest thatSphK2 expression may influence the balance between cytostasis and apoptosis.

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Introduction

Sphingolipid metabolism is a dynamic and tightly regulated process resulting in the formation of a number of bioactive metabolites, including ceramide, sphingosine, and S1P, which have all been implicated as important components of cell fate decisions (1,2). Ceramide and sphingosine are usually associated with apoptosis and negative effects on cell growth and survival, whereas, S1P opposes these effects (1). Increased ceramide and sphingosine levels have been shown to induce apoptosis in many cell types; for example ceramide and sphingosine enhance apoptosis of radiation-resistant prostate and breast cancer cells (3). In contrast, it has been shown that exogenous addition of S1P protects oocytes *in vivo* from radiation-induced apoptosis (4). This has led to the proposal that the balance between the cellular concentrations of ceramide and sphingosine versus S1P, the "sphingolipid rheostat", is important in determining whether cells survive or die (5). In agreement, studies from our lab show that sphingosine is involved in mitochondria-mediated apoptotic signaling induced by doxorubicin in human breast cancer cells (6). Whereas in sharp contrast, S1P, formed by phosphorylation of sphingosine by SphKs, protects against ceramide-mediated apoptosis and promotes estrogen-dependent tumorigenesis of breast cancer MCF7 cells (7). Despite intensive research, however, the molecular mechanisms that mediate the actions of sphingolipids in apoptosis remain unclear.

It is hypothesized that the susceptibility of cancer cells to apoptosis can be altered by modulating levels of sphingolipids. As is typical of signaling molecules, intracellular levels of S1P are low and tightly regulated; SphK plays a major role in this regulation. In contrast to SphK1, much less is known about SphK2. Elucidating the mechanism of action of SphK2 in regulating growth and apoptosis of cells may increase our understanding of the roles of sphingolipids in cancer and chemotherapeutic drug resistance. The ability to alter susceptibility of cancer cells to apoptotic stimuli may provide the basis for enhancing existing therapeutic approaches. The purpose of this study was to investigate the role of overexpressed SphK2 in cell survival and apoptosis. Furthermore, to investigate the functions of endogenous SphK2 (by downregulating SphK2) in human breast carcinoma cells and how it influences the balance between cytostasis and apoptosis in response to chemotherapeutics.

Body

Specific Aim 1. To examine the role of the BH3 domain of SphK2 and its interaction with Bcl-2 family members in human breast cancer cell lines.

In general, the stimulation of SphK1 and subsequent formation of S1P has been associated with cellular growth and survival (8,9). In sharp contrast, when SphK2 was overexpressed in MCF7 breast cancer cells a higher percentage of apoptotic cells was observed in response to serum deprivation for 24 h compared to vector expressing cells (data was shown in the 2005 annual report). Sequence analysis revealed that both human and mouse SphK2, but not their close SphK1 relatives, contain a nine amino acid sequence reminiscent of the BH3-only domain. These pro-apoptotic members of the Bcl-2 family have sequence homology only within this amphiphathic α-helical segment that allows their interaction with pro-survival Bcl-2 family members to trigger apoptosis (10,11). Similar to the association of other BH3-only proteins, results from this laboratory indicated that overexpressed SphK2 immunoprecipitated with co-expressed Bcl-x_L in MCF7 cells (data not shown). The BH3-only domain of SphK2 is unusual in that most BH3-only domains have a glycine residue before the highly conserved aspartic acid, at least three, Bnip3, Egl-1, and Bak, do not and SphK2 is the only one with leucine in this position. Substitution of the highly conserved leucine residue in the BH3 domain of these proteins has been shown to interfere with their function (10). Thus, a similar substitution was introduced in SphK2. This L219A mutation diminished the apoptotic effect of overexpressed SphK2 in NIH 3T3 cells, but not completely (Figure 1).

In order to further characterize the role of the BH3 domain in the apoptosis-inducing ability of SphK2, further studies in the lab in which SphK2 was split after its BH3 domain, forming a 227 amino acid N-terminal fragment and a 391 amino acid C-terminal fragment, both fragments lacked catalytic activity due to fragmentation of the catalytic domain. Only the N-terminal fragment, which contained the BH3-only domain, was capable of inducing apoptosis (although less than full length overexpressed SphK2) in NIH 3T3 cells, in response to serum deprivation (data not shown).

However, it was also observed that siSphK2, but not siControl, significantly induced apoptosis (data was shown in the 2006 annual report) in MCF7 cells. Furthermore, siSphK2 also sensitized MCF7 cells to apoptosis induced by 24 h treatment with doxorubicin (data was shown in the 2006 annual report and under specific aim 3). Therefore, the results were not continued in MCF7 cells since our results presented a conundrum. It has been shown that short BH3 domain peptides can induce oligomerization of Bak and Bax releasing cytochrome *c* to sensitize mitochondrial apoptosis (12) and thus, it is possible that proteolysis of overexpressed SphK2 might induce apoptosis due to liberation of its BH3 peptide domain, which does not occur with endogenous levels of SphK2.

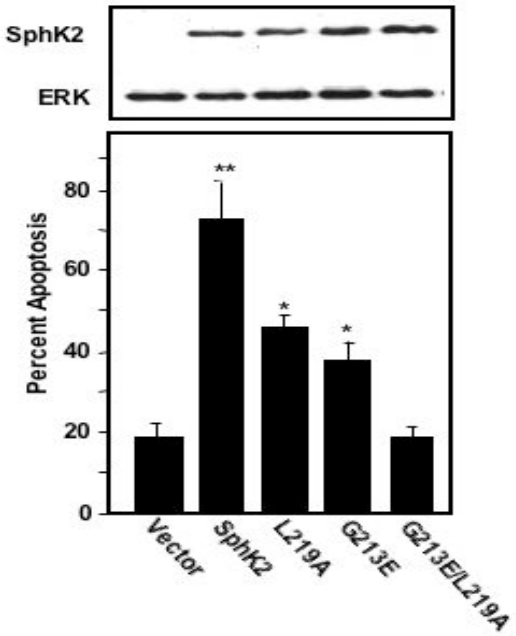

Figure 1. Overexpressed SphK2-Induced Apoptosis Requires the BH3-Only Domain and Catalytic Activity. NIH 3T3 cells were transiently co-transfected with vector, SphK2, SphK2-L219A, SphK2-G213E or SphK2-L219A/G213E with GFP at a 5 to 1 ratio. Cells were cultured in serum-free medium for 24 h, fixed, and stained with Hoechst. Total GFP-expressing cells and GFP-expressing cells displaying condensed nuclei indicative of apoptosis were scored. Each point is the mean ± SD of 3 samples. A total of at least 300 transfected cells were scored. The data are representative of at least 3 separate experiments. Equal expression of constructs was confirmed by western blotting. Lysates from duplicate cultures of NIH 3T3 fibroblasts were separated by SDS-PAGE and transferred to nitrocellulose. After blotting with anti-HA antibody to detect HA-tagged SphK2 proteins, the blot was stripped and reprobed with an anti-ERK2 antibody as a loading control.

Specific Aim 2. To determine the role of the catalytic activity of SphK2 in apoptosis of breast cancer cells.

SphK activity requires the ATP binding sequence $SGDGX_{17\text{-}21}K$, which is present within the conserved C2 domain of all the members of the SphK family (13). It has been demonstrated that a single point mutation of the second conserved glycine residue to aspartate produces a catalytically inactive SphK1 (14). In a similar manner, site-directed mutagenesis of the equivalent residue in SphK2 (G213E) resulted in a complete loss of sphingosine kinase activity. This catalytically inactive mutant was much less effective in promoting apoptosis than WT overexpressed SphK2 in NIH 3T3 cells, but it retained some pro-apoptotic activity, albeit much less than WT SphK2 (Figure 1). Notably, the double G213E/L219A mutation not only eliminated the enzymatic activity of Sphk2, it also totally abrogated its apoptotic ability (Figure 1). These effects did not result from differential expression, as the levels of the mutant SphK2 proteins were essentially the same as the WT protein (Figure 1). Collectively, these results indicate that induction of apoptosis by SphK2 overexpression requires both the catalytic activity

and the BH3-only domain of SphK2. The results were not continued in MCF7 cells for the reasons described under specific aim 1, it was therefore more important for us to examine the functions of endogenous SphK2 using small interfering RNA (siRNA) in MCF7 cells, as described in specific aim 3 and 4.

As described in the 2005 and 2006 annual report both SphK2 isoforms were found to be expressed mainly in the nucleus of MCF7 cells when examined by confocal fluorescence microscopy. Moreover, the localization of either splice variant was not affected by serum deprivation in MCF7 cells.

Specific Aim 3. To determine the effects of downregulation of endogenous SphK2 expression on the cell cycle and apoptosis.

Although experiments with overexpression of SphK2 were restricted to moderate increases in SphK2 expression, it was important to examine localization and functions of endogenous SphK2. To this end, rabbit polyclonal anti-SphK2 antibodies were utilized, which were previously used to identify endogenous SphK2 in HEK 293 cells (15), in western blotting analyses. In MCF7 cells, an immunoreactive band with the same apparent molecular mass as the endogenous SphK2 previously detected in HEK 293 cells was present in the cellular membrane fraction but undetectable in the cytosol (Figure 2). Based on initial studies in the laboratory with subcellular fractions prepared by differential centrifugation, it was observed that in contrast to previous results in HEK 293 and MDA-MB-453 cells where the majority of endogenous SphK2 was in the plasma membrane fraction (15), endogenous SphK2 in MCF7 cells was readily detected in the nuclear fraction and barely detectable in other subcellular fractions (data not shown). Because the nuclear proteins are very dilute after subcellular fractionation, more concentrated nuclear and cytoplasmic fractions were prepared with NE-PER reagents. In agreement with the confocal microscopy results (Specific aim 2), endogenous SphK2 was clearly localized in the nuclei of MCF7 cells (Figure 3). It should be noted that overexpressed SphK2-L is also detected in the cytosol fraction (Figure 3).

It has recently been suggested that SphK2-L is the predominant splice variant in several human cell lines and tissues (16). However, although mRNA for SphK2-L could be detected by quantitative PCR, endogenous nuclear SphK2 in MCF7 cells had similar electrophoretic mobility as untagged SphK2-S rather than untagged SphK2-L (Figure 3). As expected, SphK2-S migrated faster than the SphK2-L species (Figure 3). To further examine the localization and function of endogenous SphK2 in MCF7 cells, its expression was downregulated by siRNA targeted to a specific SphK2 sequence (Figure 3). It was ensured that the sequences chosen were present in both SphK2-S and SphK2-L forms. Collectively, this data suggests that the predominant form of SphK2 in MCF7 cells, which is expressed in the nucleus, is the short form of SphK2. Notably, SphK2-L is not thought to be present in mice (16).

siRNA directed toward SphK2 decreased SphK2 protein and mRNA by more than 80% without changing the level of $S1P_2$, $S1P_3$, or $S1P_5$ mRNA (data was shown in the 2006 annual report). Studies have demonstrated that in contrast to SphK1, overexpression of SphK2 suppresses growth and enhances apoptosis (17-19). Surprisingly, RNA interference to knockdown SphK2 expression inhibited glioblastoma cell proliferation more potently than SphK1 knockdown did (20).

It should be noted that MCF7 cells have lost caspase 3 expression as a consequence of a

47-base pair deletion within exon 3 of the *CASP-3* gene and show some defects in activating the apoptosis execution machinery (21). Exposure of MCF7 cells to doxorubicin for 24 h only induced minimal apoptosis (Figure 4), in agreement with previous studies (22-24). Notably, although downregulation of SphK1 itself did not induce apoptotic cell death of MCF7 cells but sensitized MCF7 cells to doxorubicin-induced apoptosis (Figure 4) (25), siSphK2, but not siControl, significantly induced apoptosis (data was shown in the 2006 annual report). Furthermore, siSphK2 also sensitized MCF7 cells to apoptosis induced by 24 h treatment with doxorubicin (data was shown in the 2006 annual report). In agreement, downregulation of SphK2 induced apoptotic traits, including activation of caspase 7, the main effector caspase in MCF7 cells, and cleavage of PARP, a marker of caspase-mediated proteolysis during the apoptotic response (data was shown in the 2006 annual report). Cleavage of the 116 kDa intact form generates an 89 kDa fragment that is used as a marker for apoptosis. Moreover, downregulation of SphK2 further enhanced doxorubicin-induced PARP cleavage, caspase 7 activation, and increased cytochrome c release to the cytosol (data was shown in the 2006 annual report), suggesting that loss of SphK2 enhances sensitivity to chemotherapy.

To characterize the involvement of endogenous SphK2 in doxorubicin-induced MCF7 cell cycle checkpoints, the effect of downregulating its expression on the cell cycle profile was examined by flow cytometry analysis (data was shown in the 2006 annual report). In agreement with several previous reports (22-24), doxorubicin treatment resulted in a marked increase in the proportion of cells in G2/M (data was shown in the 2006 annual report). Downregulation of SphK2 almost completely prevented these effects of doxorubicin (data was shown in the 2006 annual report).

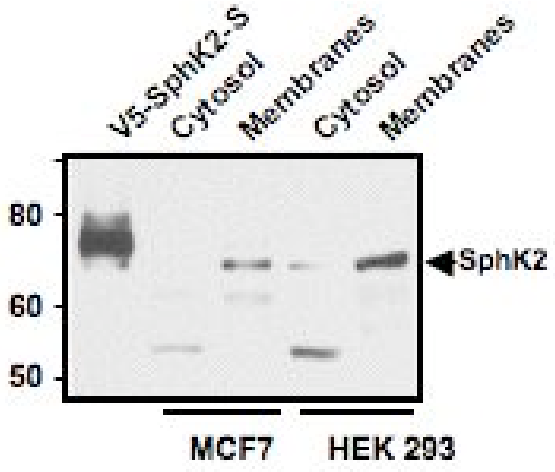

Figure 2. Comparison of Endogenous SphK2 Expression in MCF7 and HEK 293 Cells. Cytosol and membrane fractions from naive MCF7 and HEK 293 cells were prepared by centrifugation at 100,000 x g. Equal amounts of proteins were separated by SDS-PAGE and immunoblotted with anti-SphK2 antibody. Cell lysate from MCF7 cells transiently transfected with V5-tagged SphK2-S was used as a positive control.

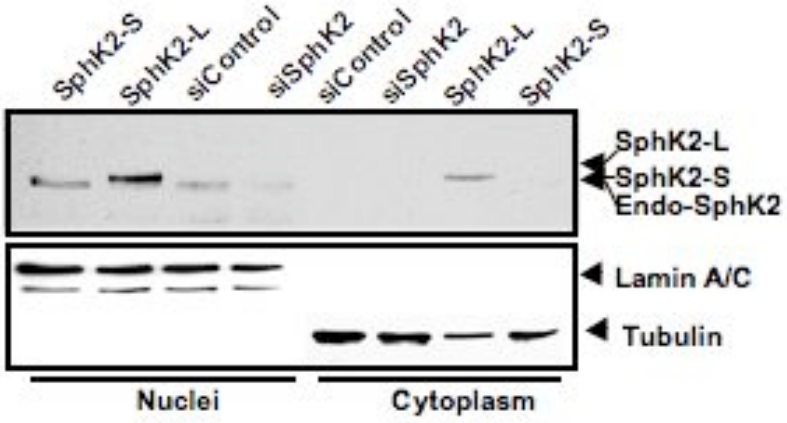

Figure 3. SphK2 is Mainly a Nuclear Protein in MCF7 Cells. MCF7 cells were transfected with control siRNA or siRNA targeted to SphK2. After 48 h, Nuclear and cytosolic fractions were prepared using NE-PER cytoplasm and nuclear extraction reagents. Equal amounts of proteins were separated by SDS-PAGE and immunoblotted with anti-SphK2. Antibodies against lamin A/C and tubulin were used as nuclei and cytoplasm markers, respectively. Cell lysates from MCF7 cells transiently transfected with untagged SphK2-S or untagged SphK2-L were included to indicate the molecular weight of these proteins. Similar results were obtained in two additional experiments.

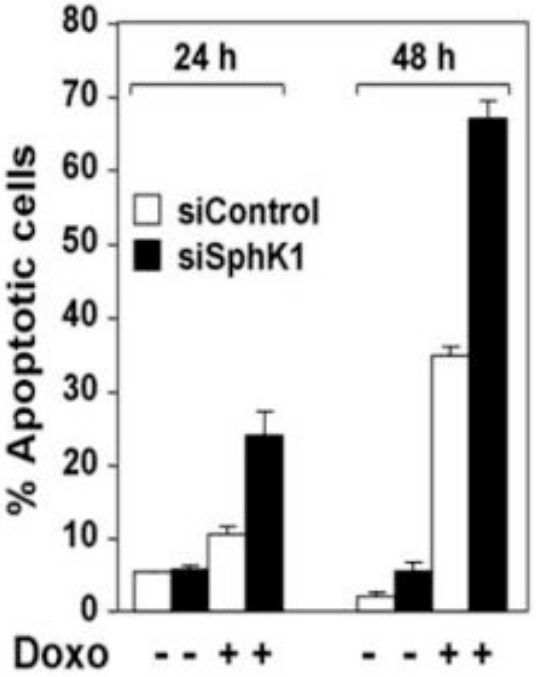

Figure 4. Downregulation of SphK1 Sensitizes MCF7 Cells to Doxorubicin-Induced Apoptosis. MCF7 cells transfected with siControl or siSphK1 were cultured in the absence or presence of doxorubicin (1.7 μM) for 48 h, fixed, and nuclei stained with Hoechst. Apoptosis was determined by scoring the percentage of cells displaying fragmented, condensed nuclei indicative of apoptosis. A minimum of three different fields were analyzed, scoring a minimum of 300 cells.

Specific Aim 4. To determine the effects of downregulation of endogenous SphK2 on signal transduction pathways.

To explore the role of endogenous SphK2 in the responses of MCF7 cells to doxorubicin, the effect of SphK2 knockdown on doxorubicin-induced p21 and p53 expression was examined. Decreasing endogenous levels of SphK2 using siSphK2 decreased basal levels of p21 and induction of p21 upon exposure to doxorubicin but had no effect on doxorubicin induced p53 expression in MCF7 cells (initial data was shown in the 2006 annual report). To exclude nonspecific off-target effects, SphK2 expression was also downregulated with siRNA targeted to two other regions of the SphK2 sequence. These siRNAs directed toward SphK2, but not scrambled siRNA controls, also markedly reduced expression of SphK2 mRNA and protein (data

not shown). They also almost completely abolished p21 expression induced by doxorubicin, without significantly altering expression of p53 (data not shown).

To further substantiate the notion that endogenous SphK2 is important for p53-independent induction of p21 by doxorubicin, MCF7 cells infected with HPV-16 E6 were utilized. Expression of HPV-16 E6 protein targets p53 protein for ubiquitination and degradation, thereby inactivating p53 function (26). In agreement with previous studies (27), there was no induction of p53 by doxorubicin in these cells (Figure 5). Nevertheless, doxorubicin induced a small increase in p21 in MCF7/E6 cells, although as expected, this response was greatly attenuated compared to MCF7 cells infected with pLXNS vector.

Downregulation of SphK2 in MCF7/E6 cells also abolished the increase in p21 induced by doxorubicin and sensitized these cells to apoptosis, as determined by cleavage of PARP (Figure 6). These results further substantiate that the effect of SphK2 on p21 is independent of p53. It should also be noted here that downregulation of SphK2 also results in a decrease in basal p21 expression and induction of p21 in response to doxorubicin in vector cells.

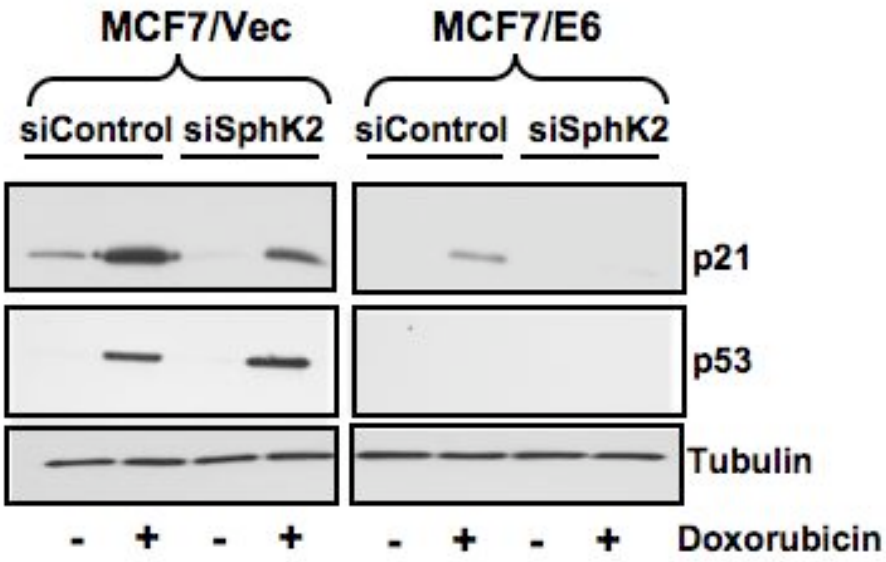

Figure 5. Effect of Downregulation of SphK2 on Doxorubicin-Induced Upregulation of p21 in MCF7 Cells Lacking p53. MCF7 infected with vector pLXSN only (MCF7/Vec) or HPV-16 E6 (MCF7/E6) were transfected with siControl or siSphK2 and cultured in the absence or presence of doxorubicin (1.7 μM) for 24 h. Equal amounts of cell lysate proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

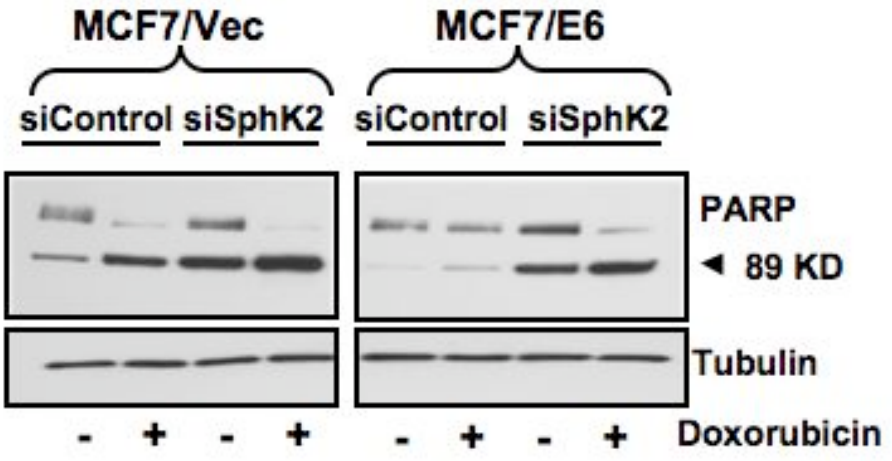

Figure 6. Effect of Downregulation of SphK2 in Doxorubicin-Induced Apoptosis in MCF7 Cells Lacking p53. MCF7 infected with vector pLXSN only (MCF7/Vec) or HPV-16 E6 (MCF7/E6) were transfected with siControl or siSphK2 and cultured in the absence or presence of doxorubicin (1.7 μM) for 24 h. Equal amounts of cell lysate proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

Materials and Methods

Reagents

Doxorubicin was purchased from Sigma (St. Louis, MO), reconstituted in molecular biology grade water, and stored protected from light. The stock solution was diluted in the relevant medium for administration to cells. Serum and medium were from Biofluids (Rockville, MD). Antibodies to p21, lamin A/C, caspase 7, phospho-p53 (Ser^{15}), and Poly(ADP-Ribose) Polymerase (PARP) were purchased from Cell Signaling (Beverly, MA). Anti-p53 antibody was from Oncogene (San Diego, CA). Anti-PDI antibody was from Stressgen Biotechnologies (San Diego, CA). Anti-tubulin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). SphK2 rabbit polyclonal antiserum raised against a unique SphK2 peptide sequence (QALHIQRLRPKPEARPR) (Biosynthesis, Lewisville, TX) was purified on a protein A column followed by affinity purification on a Sulfolink gel conjugated with the antigenic peptide according to the manufacturer's instructions (Pierce, Rockford, IL). Secondary antibodies were from Jackson Immunoresearch (West Grove, PA).

Cell Culture and Transfection

MCF7 human breast carcinoma cells were grown in phenol red-free improved minimal essential media (IMEM) supplemented with 0.25% glucose and 10% heat-inactivated FBS. Cells were cultured at 37°C and 5% CO_2. Cells were transfected using LipofectAMINE/PLUS reagent

(Invitrogen, Gaithersburg, MD) according to the manufacturer's instructions. Transfection efficiency was approximately 50%.

SphK2 expression in MCF7 cells was downregulated by transfection with sequence specific siRNA for human SphK2 (sense, 5'-GGAUUGCGCUCGUCGCUUUCAU-3'; antisense, 5'-AUGAAAGCGAGCGCAAUCCTG-3', Ambion) and control siRNA (Ambion) using Oligofectamine (Invitrogen, Gaithersburg, MD), according to the manufacturer's instructions. In some experiments, siRNA targeted to another human SphK2 sequence (5'-GCTGGGCTGTCCTTCAACCT-3', Qiagen, Valencia, CA) and control siRNA (Qiagen, Valencia, CA) was utilized. In addition ON-TARGETplus SMARTpool siRNA against SphK2 and control siRNA from Dharmacon (Lafayette, CO) were used to confirm lack of off-target effects.

Western Blot Analysis

Unless otherwise indicated, cells were lysed in buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 2 mM sodium orthovanadate, 4 mM sodium pyrophosphate, 100 mM NaF, and 1:500 dilution of protease inhibitor mixture (Sigma, St Louis, MO).

Equal amounts of proteins were separated by SDS-PAGE and transblotted to nitrocellulose, blocked with 5% non-fat dry milk for 2 h at room temperature, and then incubated with primary antibodies overnight. Appropriate horseradish peroxidase-conjugated secondary antibodies were added in Tris-buffered saline containing 5% non-fat milk. Immunoreactive signals were visualized by enhanced chemiluminescence (Pierce, Rockford, IL) and blots exposed to Kodak X-Omat film

Cell Death Assays

Apoptotic cell death was measured by staining cell nuclei with 8 μg/ml Hoechst dye 33342 bisbenzimide (Sigma, St. Louis, MO) and apoptotic cells were identified by condensed, fragmented nuclear regions using a Nikon TE 300 fluorescent microscope. A minimum of 300 cells were scored.

Statistical Analysis

For each experiment, data from triplicate samples were calculated and expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed using Microsoft Excel statistical software.

Key Research Accomplishments

(1) SphK2 overexpression enhances apoptosis in cells independently of S1P receptors.

(2) SphK2 contains a putative “pro-apoptotic” BH3-like domain.

(3) Apoptosis induced by SphK2 overexpression requires its catalytic activity and the BH3-only domain.

(4) ER stress and uptake of calcium by the mitochondria also contribute to the apoptotic effects of SphK2 overexpression.

(5) SphK2 is predominantly localized to the nucleus of MCF7 and HCT-116 cells.

(6) SphK2 may be involved in the regulation of doxorubicin induced expression of p21 in a p53-independent manner.

(7) SphK1 expression has no effect on p21 or p53 expression.

(8) Downregulation of SphK2 decreases G2/M arrest induced by doxorubicin in MCF7 cells.

(9) Downregulation of SphK2 induces apoptosis in response to doxorubicin independently of p53 in MCF7 cells.

These results suggest that SphK2 may influence the balance between cytostasis and apoptosis of human breast cancer cells.

Reportable Out comes

Abstracts and Presentations

Sankala H, Hait NC, Paugh SW, Milstien S, Spiegel S. p53 independent regulation of p21 expression by sphingosine kinase 2. The American Society for Cell Biology 46th Annual Meeting, San Diego, CA, December 9-13, 2006.

Sankala H. Sphingosine kinase 2 regulates p21 independently of p53 in MCF7 cells. 34th Annual John C. Forbes Graduate Student Honors Colloquium, VCU, May 2006.

Sankala H, Hait NC, Paugh SW, Milstien S, Spiegel S. Sphingosine kinase 2 regulates doxorubicin-induced cell cycle arrest and p21 independently of p53 in MCF7 cells. 22nd Annual Daniel T. Watts Research Poster Symposium, VCU, October 25-26, 2005.

Sankala H, Hait NC, Paugh S, Milstien S, Spiegel S. The role of sphingosine kinase 2 in doxorubicin-induced cell cycle arrest in MCF7 cells. Integrative Cellular and Molecular Signaling Symposium, VCU, October 28-29, 2005.

Sankala H, Hait N, Elmore L, Milstien S, Spiegel S. Sphingosine kinase 2 regulates p21 in MCF7 human breast cancer cells. The American Association for Cancer Research 96th Annual Meeting. Orange County, CA. 2005.

Sankala H. The role of sphingosine kinase 2 in apoptosis of MCF7 cells. 33rd Annual John C. Forbes Graduate Student Honors Colloquium, VCU, May 2005.

Sankala H, Liu H, Goparaju S, Hait N, Maceyka M, Milstien S and Spiegel S. Sphingosine kinase type 2 regulates apoptosis. 39th Annual Southeastern Regional Lipid Conference, Cashiers, NC, November 2004.

Sankala H, Liu H, Toman R, Goparaju S, Maceyka M, Bektas M, Payne S, Milstien S, Spiegel S. Dual roles of sphingosine kinase 2 in apoptosis. Massey Cancer Center Research Retreat. VCU, November 2004

Sankala H, Hait N, Milstien S, Spiegel S. The role of sphingosine kinase 2 in apoptosis of human breast cancer cells. 21st Annual Daniel T. Watts Research Poster Symposium, VCU, October 2004.

Awards

Sidney S. Negus Award for Excellence in Biochemistry
Virginia Commonwealth University, 2005

The John C. Forbes Award for Excellence in Biochemistry
Virginia Commonwealth University, 2005

Excellence in Cancer Research Award for First Place Presentation
Massey Cancer Center Research Retreat. Virginia Commonwealth University, 2004

Publications

Sankala H, Hait N, Paugh SW, Milstien S, Spiegel S. Sphingosine kinase 2 mediates induction of p21 by doxorubicin independently of p53 in MCF-7 breast cancer cells. In preparation.

Maceyka M, **Sankala H**, Hait NC, Le Stunff H, Liu H, Toman R, Collier C, Zhang M, Satin L, Merrill AH Jr, Milstien S, Spiegel S. Sphk1 and Sphk2: Sphingosine kinase isoenzymes with opposing functions in sphingolipid metabolism. J Biol Chem. 2005 Nov 4;280(44):37118-29.

Liu H, Toman RE, Goparaju SK, Maceyka M, Nava VE, **Sankala H**, Payne SG, Bektas M, Ishii I, Chun J, Milstien S, Spiegel S. Sphingosine kinase type 2 is a putative BH3-only protein that induces apoptosis. J Biol Chem. 2003 Oct 10;278(41):40330-6.

Conclusions

Two SphK2 splice variants have been described, the originally reported 68 kDa form, designated SphK2-S (28), and the N-terminal extended 72 kDa SphK2-L (16,29). Based mainly on real-time quantitative PCR of mRNA using primer sets that differentiate between SphK2-S and SphK2-L, it has been suggested that SphK2-L is the predominant form in human cell lines and tissues (16). However, the presence of endogenous SphK2 protein forms was not directly examined in these studies (16). Using immunoblotting with an antibody directed against a peptide sequence present in both SphK2-S and SphK2-L, an immunopositive band that was downregulated by siSphK2 that had a similar electrophoretic mobility as SphK2-S rather than SphK2-L was detected. These results clearly indicate that the endogenous form of SphK2 expressed in MCF7 cells, which is particularly enriched in the nucleus, is not SphK2-L.

Overexpression of SphK2 increased p53-independent expression of p21 and hypophosphorylation of Rb in MCF7 cells. In contrast, overexpression of SphK1 had no effect on either p21 or p53. Importantly, downregulation of endogenous SphK2 in MCF7 cells markedly reduced doxorubicin-induced p21, without affecting p53 expression. Although the function of SphK2 in the nucleus is not clear, we found that endogenous SphK2 is involved in doxorubicin-induced expression of p21 in a p53-independent manner.

The mechanisms that regulate p21 expression fall into two general categories: those that are either dependent or independent of the tumor suppressor, p53. Induction of the p21 gene by p53 is mediated by transcriptional activation via cis-elements located 1.95 and 2.85 kb upstream from the transcriptional start site in the mouse and 2.4 kb in the human p21 gene (30,31). p53-independent mechanisms of p21 induction and growth arrest have been less extensively studied. Emerging data has identified a variety of other transcription factors and signaling molecules that can regulate levels of p21 independently of p53 (32). A variety of transcription factors, including STATs, E2Fs, AP2, C/EBPα, C/EBPβ, and the homeobox transcription factor gax, can regulate p21 trancription through *cis*-acting elements in the p21 promoter (33). p21 expression may also be regulated posttranscriptionally by both ubiquitin-dependent and -independent proteasome-mediated degradation (34,35).

p21 appears to be a major determinant of cell fate in response to anticancer therapy as it plays an essential role in growth arrest after DNA damage (36,37), and can function to inhibit p53-dependent and -independent apoptosis (23). Thus, repression of p21 by gene targeting, c-Myc or chemical p21 inhibitors, all sensitize tumor cells to apoptosis by anticancer drugs (24,38). Indeed, downregulation of SphK2 inhibited doxorubicin-induced G2 checkpoint arrest in MCF7 cells, consistent with the failure of these cells to up-regulate p21 in response to doxorubicin. Importantly, it also sensitized them to apoptosis induced by doxorubicin with a concomitant activation of caspase 7 and PARP cleavage, suggesting that endogenous SphK2 may regulate sensitivity to chemotherapy. Thus, similar to SphK1 (25), SphK2 may also be important for growth and survival of MCF7 cells.

According to this idea, doxorubicin-induced expression of p53, which in turn can induce p21, leads to cell cycle arrest, or induction of pro-apoptotic factors, including Puma and Noxa, leading to cell death (24). SphK2 is required for maximal increases in p21 (independently of p53), enabling cell cycle arrest, DNA repair, and preventing execution of the cell death program. Downregulation of SphK2 expression, represses p21 and switches the response from cell cycle arrest to apoptosis, suggesting that SphK2 may influence the balance between cytostasis and

apoptosis of human breast cancer cells. Thus, targeting SphK2 to decrease p21 expression may have the potential to improve the action of anticancer drugs.

Collectively, our results raise the conundrum of how overexpression of SphK2 decreases cell growth and enhances apoptosis, while its downregulation sensitizes cells to apoptosis. However, other reports of this phenomenon have been shown in the literature, Daxx protein (an essential protein found in mice) for example. Overexpressed Daxx was shown to induce apoptosis. However, increased apoptosis was observed in Daxx knockout embryos. siRNA studies confirmed that endogenous Daxx functioned as a pro-survival protein rather than a pro-apoptotic protein, as initially thought (39). Similarly, our studies suggest that endogenous SphK2 functions as a pro-survival protein.

Additionally, similar to our results with siSphK2 a study by Van Brocklyn *et al.,* found that decreasing either SphK2 or SphK1 expression by RNA interference decreased growth of U-1242 MG and U-87 MG glioblastoma cells. Surprisingly, RNA interference to knockdown SphK2 expression inhibited glioblastoma cell proliferation more potently than SphK1 knockdown did (20). Together these results suggest that similar to SphK1 (25), SphK2 may also be important for growth and survival of cancer cells.

Importantly, although neither SphK1 nor SphK2 null mice have an obvious phenotype or marked deficiency of S1P, the double knockouts completely lack S1P and have severely disturbed neurogenesis, including neural tube closure, and angiogenesis defects, resulting in early embryonic lethality before day 13.5 because of severe bleeding (40). Thus, it seems that SphK1 and SphK2 can complement for the loss of one of the isozymes, and during development S1P formation is absolutely critical.

A partial answer may also be provided by results showing that when SphK2 is overexpressed it does not always have the same subcellular distribution as the endogenous protein. Results from our laboratory showed that overexpressed SphK2 was localized to the ER in NIH 3T3 cells, in addition targeting pro-survival SphK1 to the ER, also resulted induced apoptosis in response to serum withdrawal (data not shown). Alternatively, SphK2 contains an α-helical BH3 domain analogous that present in other BH3-only pro-apoptotic proteins (17), such as Bim and Bid. It has been shown that short BH3 domain peptides can induce oligomerization of Bak and Bax releasing cytochrome *c* to sensitize mitochondrial apoptosis (12) and thus, it is possible that proteolysis of overexpressed SphK2 might induce apoptosis due to liberation of its BH3 peptide domain.

Clarifying the functions of SphK1 and SphK2 has important implications in terms of anticancer therapeutics targeting. A broad specificity inhibitor of SphKs may be counterproductive if SphK2 has negative growth-regulatory effects. However, specific inhibitors of SphK1 may not be sufficient to decrease metastasis if SphK2 can compensate. With this in mind interestingly, non-isospecific inhibitors of SphK, such as DMS and DHS induce apoptosis (41), which may be a further indication that endogenous SphK2 has pro-survival effects.

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